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Axon guidance effect of classical morphogens Shh and BMP7 in the hypothalamo-pituitary system

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HIGHLIGHTS

- BMP7 and Shh are expressed in the anterior and posterior ventral midline of the hypothalamus.
- *Shh*+ anterior and *Bmp7*+ posterior ventral midline repel hypothalamic axons.
- Shh and BMP7 mediate the repulsive effects of the hypothalamic ventral midline.

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ABSTRACT

Hypothalamus plays a key role in homeostasis, and functions of the hypothalamus depend on the accurate trajectory of hypothalamic neuroendocrine axons. Thus, understanding the guidance of hypothalamic neuroendocrine axons is crucial for knowing how hypothalamus works. Previous studies suggest FGF10 deriving from the medial ventral midline of the hypothalamus plays an important role in axon guidance of the developing hypothalamus. Here we show that Shh and BMP7, which are from the anterior and posterior hypothalamic ventral midline respectively, together repel hypothalamic axons towards the medial ventral midline.

1. Introduction

In the developing nervous system, axon guidance depends on extracellular matrix cues deriving from cells along the pathway, and on gradients of chemoattractant/chemorepellant molecules emanating from more distant cellular sources [6,9,36]. From 1990s, Netrins, Slits, Semaphorins and Ephrins have been identified as four major conserved axon guidance cues [9,30]. However, these four classical families of guidance cues cannot explain all the guidance events. In addition to these four major conserved families of guidance cues, other molecules have been implicated in axon guidance. In particular, families of morphogens have been reported to play an important role in regulating axon guidance. Morphogens are secreted proteins produced by restricted groups of cells, and provide positional information by long-range concentrations [29,35]. Recently, morphogens with evolutionarily conserved roles in patterning embryonic tissues, such as Hedgehogs (Hhs), bone morphogenetic proteins (BMPs), Wnts and fibroblast growth factors (FGFs) have been shown to act as guidance cues [5,28]. The patterning function of such morphogens begins at very early stages of development, so it is reasonable to speculate that the remaining established gradient of these morphogens might provide directional information for axons along body axes at later stages [38]. Here, our studies are consistent with the emerging idea that classical morphogens are potent axon guidance cues, demonstrating that Shh and BMP7 can act as guidance cues to direct hypothalamic neurosecretory axons into the medial ventral midline (MVM) of the hypothalamus.

2. Materials and methods

2.1. In situ hybridization

Embryos were processed for in situ hybridization as described previously [33]. Following development, embryos or explants (minimum 5 in each condition) were analyzed as whole-mount preparations or cryostat sections.

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2.2. Explant culture

All embryos were staged and dispase-isolated (1 mg/ml, Roche). According to the requirements of distinct experiments, different parts of the hypothalamus were dissected out. Explants were then cultured in collagen beds based on published techniques [25]. When two explants were cultured together, the distance between them was around 100–300 μm.

2.3. Tracing of neural projections with DiI

The lipophilic carbocyanine dye, DiI (Molecular Probes) was injected into the MVM in open-book preparations, explants cultured to E6 and fixed in 4% paraformaldehyde.

2.4. Protein use

Proteins (BMP7, Shh, and chordin) were obtained and used as described [22]. Proteins were tested in one of two ways: by soaking to beads, or by adding directly to culture medium. Proteins ((BMP7, Shh) were pre-soaked on Affigel beads (Pharmacia Biotech). In co-cultures, beads were positioned approximately 300 μm from the explants. Protein chordin or Shh blocking antibody, 5E1 (20 μg/ml; [10]) was added to the culture medium at the start of incubation.

2.5. Immunofluorescence analyses

Immunohistochemical analysis of explants was performed according to standard whole-mount or cryostat sectioning techniques [23]. Antibody anti-Neurofilament (3A10; DSHB) was used. Secondary antibodies were conjugated to Alexa 594 or Alexa 488 (Molecular Probes).

2.6. Statistical analyses

Axon numbers were recorded after 48 h, and data were analyzed using GraphPad Prism 4.0 for PC. Statistical significance of differences in means between groups was determined using the students paired *t*-test or unpaired *t*-test. *P* values less than 0.05 were considered significant for our analyses.

3. Results

3.1. Shh and Bmp7 expression during the innervation of the developing hypothalamus

As described previously, hypothalamic axons respond to guidance cues at E4-E5, project to the area of MVM at E6 [17]. We first characterized the morphology of the hypothalamus by analyzing expression of *Shh and Bmp7* at E5. *Shh* expression shows that, in contrast to its usual restricted midline expression, *Shh* is expressed in anterior ventral midline (AVM) and lateral cells of the hypothalamus (Fig. 1A–C). *Bmp7* has been reported to be expressed in the ventral midline of the hypothalamus at early stages (E2-3) [22,18]. Our results reveal that *Bmp7* is only expressed in the posterior ventral midline (PVM) (Fig. 1D–F).

To further analyze the growth of axons, we performed retrograde DiI-labelling experiments ($n = 5$) into 'open book' hypothalamic explants. DiI, the lipophilic carbocyanine dye, dissolves in the lipid layer of the plasma membrane and diffuses anterogradely and retrogradely along neuronal processes [12]. The hypothalamus was dissected out in an 'open-book' flat configuration, so that anterior, medial and posterior portions of the ventral midline can be distinguished morphologically (Fig. 1G). DiI was injected into the MVM domain of the explants (schematized in Fig. 1H), and explants were cultured for 2 days, until the equivalent of E6. Long axons/fascicles emerge and project into the MVM (Fig. 1I).

3.2. Chemorepulsive effects of AVM and PVM

Throughout the posterior neural axis, the floor plate is known to play an important role in guiding spinal axons [6]. Studies have shown that the ventral midline of the hypothalamus shares an origin with floor plate cells of the hindbrain [18], which raises the possibility that the ventral midline of the hypothalamus might also influence the growth of hypothalamic axons. One study has suggested that in the developing hypothalamus, GnRH (gonadotropin-releasing hormone) axons follow a diffusible chemoattractive cue(s) secreted by the MVM to reach their target cells [27].

To directly address whether the hypothalamic ventral midline can guide axons, we performed 3-d collagen co-cultures, a well established method to detect long-range chemotropic and chemorepulsive activities [31,26]. To provide stronger evidence for this hypothesis, the hypothalamic ventral midline was subdissected into anterior and posterior portions. Midline explants were then cultured at a short distance from lateral explants for 2 days. Robust axon outgrowth is elicited by the midline explants (Fig. 2Ai, Bi and Ci); and few axons emerge from lateral explants cultured alone (Table 1; Fig. 2D). In marked contrast to the robust attraction provoked by MVM explants [17], AVM and PVM explants both mediate a repulsion of hypothalamic axons (Fig. 2Ai, Bi and Ci) ($n = 5$, 11 respectively), with significantly more axons projecting away from either PVM or AVM explants (Fig. 2E, F; Table 1). This suggests that both the AVM and PVM may exert a chemorepulsive effect on axons. The accuracy of dissection confirmed through in situ analysis of *Shh* and *BMP7*, respectively (Fig. 2Aii, Bii and Cii).

Together, these results suggest that the *Shh+* AVM and *Bmp7+* PVM repel hypothalamic axons, by contrast to the chemoattractive effects of the *Fgf10+* MVM [17].

3.3. Shh/BMP7 mediate the repulsive effects of the ventral midline

As described above, morphogens can contribute to axonal pathfinding in various systems. Morphogen FGF10 deriving from the MVM of the chick hypothalamus plays an important role in guiding hypothalamic axons to turn into the *Fgf10+* MVM at E4-E6 [17]. Our analyses suggest that *Bmp7* and *Shh* are expressed in distinct regions of the chick hypothalamic midline at E4-6, which raises the possibility that morphogens BMP7 and Shh might be responsible for the repulsive effects of the midline hypothalamus.

To establish whether Shh might be a repellant cue for hypothalamic axons, lateral explants were dissected out from E4 embryos, and then cultured with Shh-soaked beads in collagen gels. Like the AVM of the hypothalamus, Shh-soaked beads repel hypothalamic axons in vitro ($n = 7$) (Fig. 3A and 3Ei; Table 1). Moreover, the Shh blocking antibody 5E1 effectively blocks the chemorepellant function of Shh ($n = 7$) (Fig. 3C and Eiii; Table 1). All these observations indicate that Shh deriving from the lateral hypothalamus and the AVM of the hypothalamus act as a repellant guidance cue for hypothalamic neurosecretory axons.

To further test the hypothesis, we examined the response of lateral explants from E4 chick embryos to a BMP7 gradient, the source of BMP7 provided by an Affigel bead loaded with recombinant protein. BMP7 beads elicit robust outgrowth of hypothalamic axons, but these axons appear to grow away from the BMP7 source ($n = 7$) (Fig. 3B and Eii; Table 1). Chordin is a BMP inhibitor whose function is to bind the extracellular BMP ligands and prevent the interaction between the ligands and their receptors [24,8]. In the presence of chordin, the axon repellant activity induced by BMP7 is blocked ($n = 5$; $n = 7$) (Fig. 3D and Eiv; Table 1). This result supports

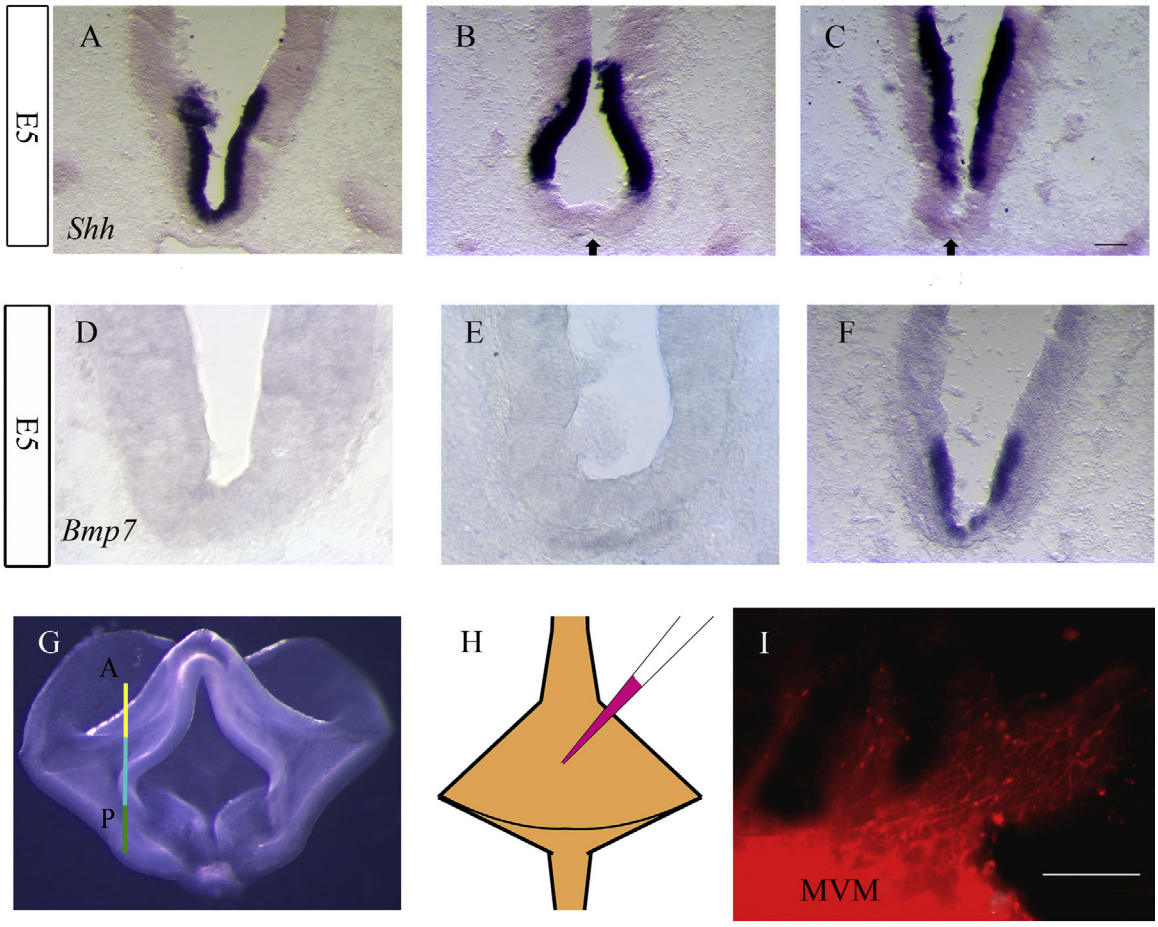

Fig. 1. Chick hypothalamic axons project to the MVM. (A–C) In situ hybridization on transverse sections at E5 reveals that *Shh* is expressed in the ventricular zone of the lateral hypothalamus and the AVM; *Shh* is not expressed in the MVM and PVM (arrows in B and C). (D–F) BMP7 mRNA is detected in the PVM of the hypothalamus, while there is no obvious expression in the anterior and medial hypothalamus. Scale bar: 100 μm. (G and H) Entire hypothalamus was dissected out from E4 chick embryos, and DiI (red) was injected into the MVM. (I) Tracing with DiI shows that axons of the hypothalamic neurons enter into the MVM. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

our hypothesis that BMP7 possesses chemorepellant activity for hypothalamic axons.

Expression of the mature neuronal marker Neurofilament (3A10) was then examined on lateral explants ($n = 5$ explants each). As remarked in other studies, when lateral explants cultured alone, axons remain confined to the explant [31]: axons extend to the edge of the lateral explant, then circle around the peripheral component, instead of extending into the collagen gel (Fig. 3F). However, Shh or BMP7 beads elicit axons growing out, and strong immunolabelling of Neurofilament appears on both cells/axons within and outside the lateral explants (Fig. 3G and H). These results indicate that the Shh and BMP7 beads secret diffusible molecules that exert a long-range effect on hypothalamic axons.

To examine evidence for a chemorepellant effect of BMP7 and Shh for endogenous hypothalamic axons, we implanted Shh or BMP7 beads ectopically into the MVM of E4 'open book' hypothalamic explants (Fig. 3J and K), and cultured for 2 days. In control group (with a PBS bead implanted in the MVM) (Fig. 3I), hypothalamic axons, labelled by 3A10, all project into the MVM. When Shh-soaked beads were implanted in the MVM, the extension of hypothalamic axons is prevented at a defined distance from the MVM and axons fail to project beyond the lateral hypothalamus

Table 1
Statistical analyses between groups and statistical analyses between axons in different regions (section i and section ii). The number of axon bundles (mean ± SEM) in section i (towards co-cultures, proximal region) and in section ii (away from co-cultures, distal region) are shown. Statistical comparisons between groups (co-cultured groups versus LE cultured alone group) and statistical comparisons between axons in different regions (section i versus section ii) are also shown.

Groups	*n*	Number of axon bundles		vs LE cultured alone		Section i vs Section ii	
		Section i	Section ii	*T* (1)	*P*	*T* (2)	*P*
LE + PVM	11	4.273 ± 0.7273	26.36 ± 3.043	6.989	<0.0001	8.302	<0.0001
LE + BMP7 bead	7	6.857 ± 1.438	26.14 ± 1.908	11.78	<0.0001	7.586	0.0003
LE + BMP7 bead + chordin	5	4.200 ± 0.9695	5.400 ± 0.8718	2.327	0.0355	1.809	0.1447
LE + AVM	5	2.600 ± 0.6782	25.00 ± 3.033	8.459	<0.0001	6.766	0.0025
LE + Shh bead	7	2.429 ± 1.020	17.29 ± 3.205*	4.628	0.0003	4.934	0.0026
LE + Shh bead + 5E1	7	4.000 ± 1.091	4.286 ± 0.9689	1.913	0.0738	0.2289	0.8265

SEM, standard error of the mean; LE, lateral explant; *n*, number of lateral explants analyzed, *T* (1), unpaired *t*-test score; *T* (2), paired *t*-test score. Number of axon bundles in LE cultured alone group is: 4.182 ± 1.354.

* Comparison between LE + BMP7 bead (section ii) and LE + Shh bead (section ii) ($P = 0.0351$, $T = 2.375$; T = unpaired *t*-test score).

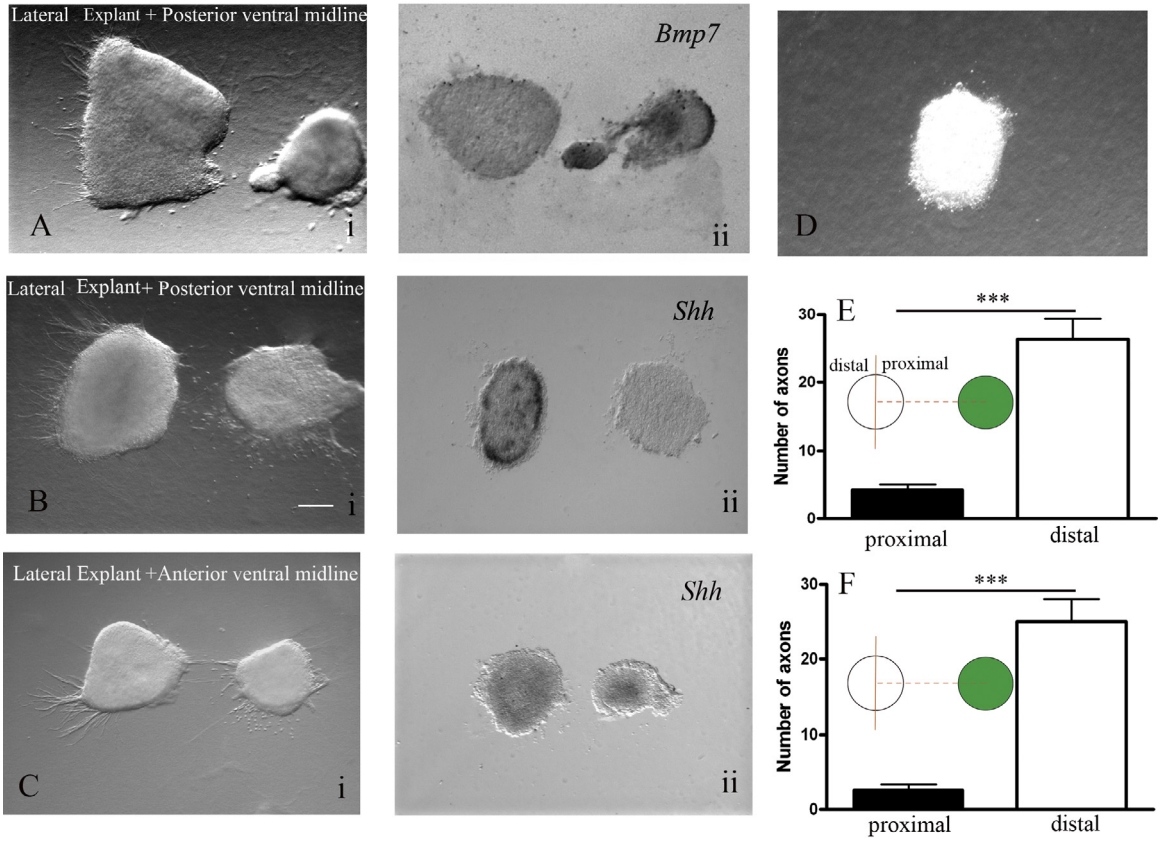

Fig. 2. AVM and PVM of the hypothalamus repel hypothalamic axons. (A–C) Co-culture of the PVM (Ai, Bi) or the AVM (Ci) with the lateral explants shows that the ventral midlines extensively promote axon outgrowth, and these axons appear to be repelled. (Aii) *Bmp7* is positive in the explants of the PVM. (Bii, Cii) Lateral explants and the AVM express *Shh*. (D) Minimal axons grow out when lateral explants cultured alone. (E, F) Significantly more axons extend from distal face versus proximal face of lateral explants (***$P < 0.001$). Error bars represent SEM. Scale bar: 100 μm.

(Fig. 3J). A different effect is provoked by BMP7 beads: a large portion of axons avoid and turn away from the MVM (Fig. 3K). Taken together, these data suggested that ectopic Shh and BMP7 affect the projection of endogenous hypothalamic axons, suggesting a requirement for Shh and BMP7 in early hypothalamic axon guidance.

4. Discussion

Hypothalamo-pituitary neuraxis is established when neuroendocrine neurons located in distinct nuclei in the hypothalamus project axons, via the MVM (forming median eminence), to synapse with capillaries in either the MVM or the neural-derived posterior pituitary [19,37]. Given the important roles of the projection of hypothalamic axons to connect with the endocrine system, surprisingly little is known of the mechanisms that govern axon guidance in this region. Here, our observations add to the growing body of evidence that BMP7 and Shh, besides their well-characterized effects as morphogens, also have additional functions as axon guidance cues.

4.1. Roles of Shh and BMP7 in the chemorepulsion for hypothalamic axons

Shh and BMPs have long been reported as morphogens that regulate the dorso-ventral patterning of the neural tube [14,15,13]. However, at later stages, the remaining gradient of BMPs and Shh establishes the ventral trajectory of commissural axons in the spinal cord [1,3,4]. In the chick hypothalamus, BMPs and Shh collaborate to play key roles in regulating cell identities [22,18,7,21]. Here we show that, as in the spinal cord, the remaining gradients of BMP7 and Shh also contribute to the axon guidance in the hypothalamus. Increasing evidence indicates that both BMPs and Shh can function as either chemattractants or chemorepellants [1,20,32,4,16,2,11]. Three lines of evidence suggest that BMP7 and Shh might act as chemorepellants for hypothalamic neurosecretory axons. First, both promote an outgrowth of axons in vitro that is directed down the concentration gradient of Shh/BMP7. Second, the antagonists of BMP and Shh signalling, chordin and 5E1, respectively, can block such axon guidance effects of BMP7 and Shh. Third, in a situation that more closely matches the in vivo environment, ectopic BMP7 and Shh can interfere with the correct pathway of endogenous hypothalamic axons. The activity of BMPs and Shh in specifying cell identities in the hypothalamus therefore raises the possibility that the guidance effects of BMP7 and Shh are not due to a chemorepellant function, but due instead to their morphogenic effects. However, our ectopic BMP7/Shh experiments go some way towards proving a direct effect of BMP7 and Shh on hypothalamic axons. Moreover, in the chick model, BMPs and Shh govern the patterning of the developing hypothalamus at relative early stage (before E5) [7,18,21,22]. Interestingly, recent studies have shown that the reorientation of axons by BMP7 or Shh is distinct from its ability to induce distinct neurons. Shh and BMP7 were found to act directly on developing axons without evoking transcription within neuron cells [32,34]. In sum, these data strongly suggest the chemorepulsive effect of BMP7 and Shh on hypothalamic axons.

Our in vitro experiments demonstrate that BMP7 and Shh act directly on hypothalamic axons to repel these axons into the median eminence. However there are differences between the

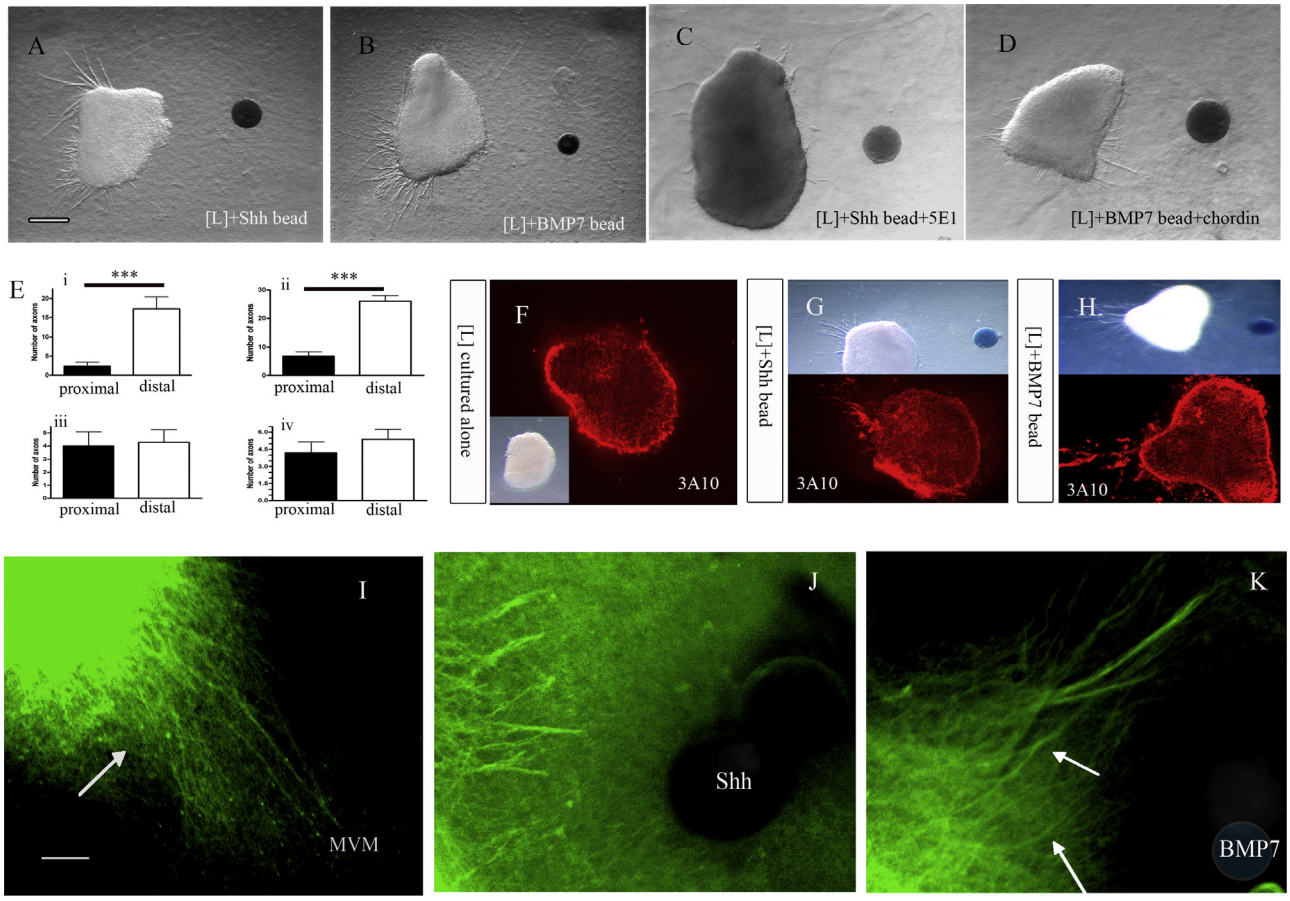

Fig. 3. Shh and BMP7 orient hypothalamic axons. (A, B) Shh and BMP7 beads repel hypothalamic axons away. (C, D) Treatment with 5E1 or with chordin abolishes the axon repulsive effect. (E) (Ei, Eii) Quantitative analyses show that the number of axons in distal section is significantly more than the number of axons in proximal section; (Eiii, Eiv) No statistical difference between axons in distal and in proximal sections. Neurofilament (3A10) expression is detected in lateral explants cultured alone (F), and also detected when cultured with Shh (G) or BMP7 beads (H). [L]: lateral explants. Scale bar: 100 μm. 'Open-book' hypothalamic explants cultured under control conditions or with ectopic Shh or BMP7 beads. 3A10 expression is detected. (I) In the control group, hypothalamic axons (arrow, I) grow into the MVM. (J) With Shh beads in the MVM, hypothalamic axons are stalled in the lateral hypothalamus. (K) With BMP7 beads in the MVM, hypothalamic axons (arrows, K) turn away from the MVM. Scale bar: 50 μm.

chemorepulsive effects of BMP7 and Shh. Our data indicate that more hypothalamic axon bundles emerge to be repelled by BMP7-soaked beads than Shh beads ($P = 0.0351$; Table 1), since more axons appear to form axon fascicules/bundles in the presence of Shh. Ectopic bead implant experiments indicate that Shh can induce the formation of axon fascicles and prevent axons from growing into sources of Shh protein; by contrast, hypothalamic axons simply appear to be repelled from BMP7 sources (compare Fig. 3J and K). The differences mediated by Shh and BMP7 add new insights into our understanding of axon guidance in the hypothalamus. As observed in Fig. 1, *Shh* is expressed in the ventricular zone of the lateral hypothalamus. Our results suggest that the chemorepulsive effect of Shh repels hypothalamic axons from the ventricular zone of the hypothalamus and ensure that these axons travel through the mantle layer of the hypothalamus in fascicules.

4.2. A model system for hypothalamic axon guidance

Here our in vitro studies provide significant insight into the mechanisms of axon guidance within the developing chick hypothalamus. These data are consistent with the emerging idea that classical morphogens are potent axon guidance cues. Finally, our data with observations obtained before [17] suggest a model system for hypothalamic axon guidance. In this model, FGF10 is a pivotal factor that attracts both hypothalamic axons and vascular endothelial cells into the forming median eminence; Shh serves initially to induce the formation of fasciculated hypothalamic axons and directs their growth along the mantle layer, then collaborates with the chemorepellant BMP7 preventing hypothalamic neurosecretory axons from entering into other inaccurate domains of the hypothalamus, and repelling neurosecretory axons into the MVM of the hypothalamus.

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